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(54) Title: VACCINES FOR ORAL IMMUNIZATION AGAINST INFECTING AGENTS

(57) Abstract

The invention relates to conjugates of an antigenic material selected from the group of a toxin, or a fragment thereof, a toxoid and/or an adherence antigen derived from an infecting agent, wherein said antigenic material is covalently bound to a physiologically acceptable inert carrier, such as silica, chemically-modified silica, aluminium silicate, kaolin or latex. The conjugates are for use in vaccines for oral immunization against infecting agents, e.g., gastrointestinal microbial infections such as cholera.

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VACCINES FOR ORAL IMMUNIZATION AGAINST INFECTING AGENTS

Field of the Invention

The present invention relates to vaccines for oral immunization comprising a conjugate of a toxin covalently bound to a physiologically acceptable inert carrier in microparticulate form, to said conjugates and to a method for their production.

Background of the Invention

Vaccines for oral immunization against several bacterial infections have been disclosed in recent years. Most of these vaccines comprise an attenuated microorganism, such as typhoid vaccine comprising an attenuated mutant of Salmonella typhi Ty 21a in sodium bicarbonate or in enteric-coated capsules (Black, R. et al., 1982 and 1983), or a lyophilized live Salmonella typhi 21a vaccine in capsules (Mascart-Lemone, F. et al., 1988), or lyophilized recombinant V. cholerae CVD 103 or CVD 103-HgR microorganisms resuspended in sodium bicarbonate (Levine, M.M. et al., 1988).

Staphylococcal enterotoxin B has been encapsulated in microspheres from biodegradable polymers of lactic acid and glycolic acid and used as primary and booster immunizations given orally or intramuscularly (Edelman, R., 1989). A purified glutaraldehyde-treated cholera toxoid was suspended in distilled water alone or containing sodium bicarbonate and then administered orally to volunteers (Levine, M.M. et al., 1978).

Inert carriers bonded to chemical drugs are described in European Patent Application EP 305968, published on March 8, 1989, which discloses pharmaceutical compositions against protozoal diseases comprising micron-size particles of physiologically acceptable natural particles selected from silica, aluminum silicates, kaolin, etc. chemically bonded to an anti-protozoal drug. These particles are not used for immunization, but for killing the pathogen. They are ingested by the protozoa, e.g. amoeba, and the active drug is released thus causing the death of the protozoa.

Summary of the Invention

It has now been discovered that when a toxin, a fragment thereof, a toxoid and/or an adherence antigen material derived from a target infecting agent is covalently bound to a physiologically acceptable inert carrier in microparticulate form, conjugates which are efficient for oral immunization against said infecting agents are obtained.

It is thus an object of the present invention to provide such conjugates suitable for oral immunization.

Another object of the invention is to provide a method for the production of said conjugates.

In another embodiment, the invention relates to vaccines for oral immunization comprising the conjugates of the invention.

A further object of the invention is to provide a method for oral immunization by administering the vaccines of the invention.

Brief Description of the Drawings

Fig. 1 shows the detection of IgA antitoxin antibodies in the ileal secretion of rabbits after administration of a single dose of cholera toxin B subunit linked to a silica carrier.

Fig. 2 shows the ELISA of sIgA of ileal secretions from two groups of three rabbits that underwent Thiry-Vella surgical modification and which were primed with 0.5 mg cholera toxin B subunit (CTB) alone or linked to silica (Si-CTB).

Fig. 3 shows the challenge of rabbits immunized with silica-bound cholera toxin B subunit and silica alone with *V. cholerae* 01 strain 395.

Fig. 4 shows a fluorescence micrograph of a Bouin-fixed, unstained intestinal section showing characteristic embedded microparticle-like structures in Peyer's patches.

Fig. 5 shows the intestinal persistence of unlinked and silica-linked ^{125}I radiolabeled cholera toxin B subunit perorally administered to two groups of three rabbits.

Fig. 6 shows the scanning transmission image of a fixed, unstained section of an ileal section showing characteristic embedded microparticle-like structures.

Fig. 7 shows the energy spectrum from a single embedded microparticle-like structure of Figure 6.

Fig. 8 shows the ELISA of sIgA from lung lavage secretions obtained from three C57BL/10J mice primed and boosted with thyroglobulin-bound CTB peptide 3 (CTP3) linked to silica (Si-TGB-CTP3) or free (TGB-CTP3).

Fig. 9 shows the ELISA of IgG from serum obtained from five SJL/J mice primed and boosted with thyroglobulin-bound CTP3 linked to silica (Si-TGB-CTP3) or free (TGB-CTP3).

Description of the Invention

The antigens of the conjugates of the present invention are natural toxins, natural or synthetic fragments thereof, toxoids and/or adherence antigenic materials.

In a preferred embodiment, a vaccine against Vibrio cholera is prepared comprising a conjugate of cholera toxin B subunit (CTB) or a synthetic fragment peptide which consists of a portion thereof, e.g. peptide CTP 3 comprising the 50-64 amino acid sequence of the B chain of the formula,

Val-Glu-Val-Pro-Gly-Ser-Gln-His-Ile-Asp-Ser-Gln-Lys-Lys-Ala
50 60

linked to an inert carrier. Other CTB peptides, namely, CTP1, CTP2, CTP4, CTP5 and CTP6 have also been synthesized and may similarly be linked to an inert carrier. These peptides have the following sequences : CTP1 - Leu-Cys-Ala-Glu-Tyr-His-Asn-Thr-Gln-Ile-His-Thr-Leu; CTP2 - Ser-Leu-Ala-Gly-Lys-Arg-Glu-Met-Ala-Ile-Ile-Thr-Phe; CTP3 - Val-Glu-Val-Pro-Gly-Ser-Gln-His-Ile-Asp-Ser-Gln-Lys-Lys-Ala; CTP4 - Lys-Asn-Thr-Leu-Arg-Ile-Ala-Tyr-Leu-Thr-Glu-Ala-Lys-Val-Glu-Lys-Leu; CTP5 - Ala-Tyr-Leu-Thr-Glu-Ala-Lys-Val-Glu-Lys-Leu; CTP6 - Lys-Leu-Cys-Val-Trp-Asn-Asn-Lys-Thr-Pro-His-Ala-Ile-Ala-Ala. A variety of other toxins or fragments thereof of various origins can be used according to the present invention, such as for example the toxins produced by other enteric pathogens, e.g. E. coli-LT,

E. coli-STa, E. coli-STb and other E. coli strains, Salmonella, Klebsiella, Shigella, Clostridium perfringens type A, Bacillus cereus, V. parahemolyticus, Staphylococcus aureus, Clostridium difficile, Clostridium botulinum, Giardia lamblia, etc; toxins produced by pathogens associated with respiratory and pulmonary infections, e.g. Mycoplasma pneumoniae, Bordetella pertussis, Streptococcus pneumoniae, Haemophilus influenzae, etc., pathogens associated with urogenital tract infections, e.g. certain strains of E. coli, Candida albicans, Pseudomonas aeruginosa, Streptococcus viridans, etc., or pathogens associated with the buccalpharyngeal tract infections, e.g. Trichomonas tenax.

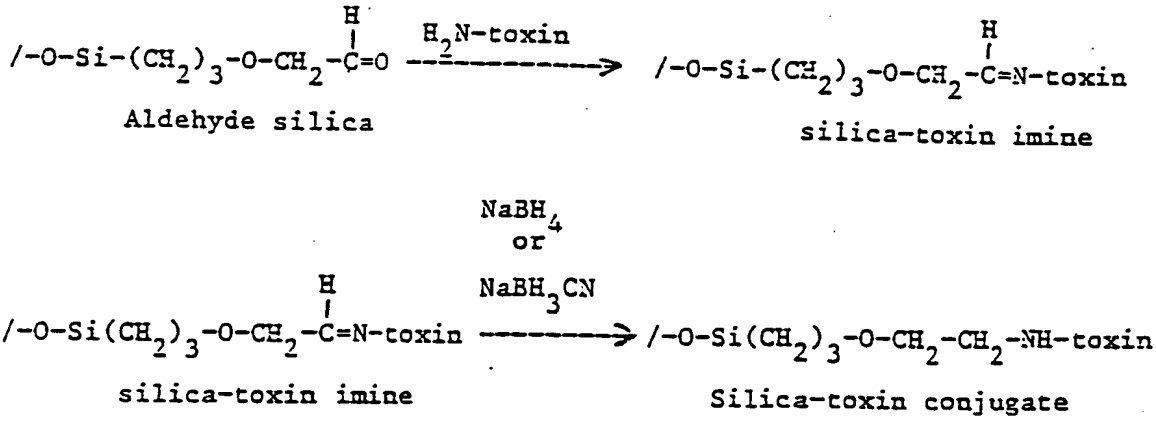
The adherence antigenic materials according to the invention include cell surface antigens derived from microbial pathogens, e.g. bacteria, yeast, fungi, such as colonization factor antigens (e.g., CFA I, CFA II, CFA IV, CS1-CS6, 0 antigens etc.), pilins (TCP), adhesins, fimbriae (K88, K89), membrane glycoproteins and the like, which enable the enteric pathogen to adhere to the intestinal mucosa, an essential prerequisite for the development of the disease. By preventing microbial adhesion to the mucosa, an effective tool for the prevention of the disease is provided.

A wide variety of inert carriers, both organic and inorganic, may be used according to the invention. Examples are microparticulates of silica, aluminum silicate, kaolin, latex, etc., which have been chemically modified so as to render them attachable to biomolecules, such as the proteinaceous antigens of the invention.

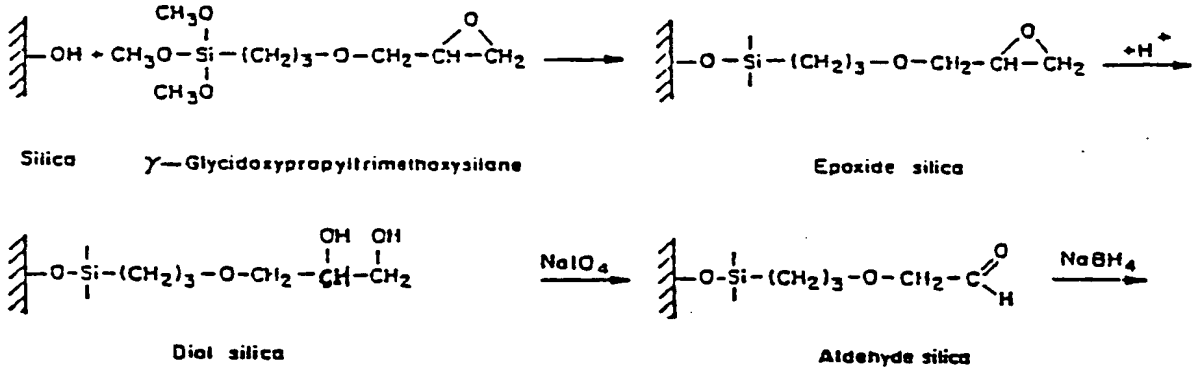
Examples of carriers that may be used in the present invention are chemically modified silicas carrying side chains with a terminal group such as OH, COOH, NH_2, CHO, SH, etc., (described by K. Ernst-Cabrera and M. Wilchek, 1988). The conjugates will be formed by the reaction of functional groups of the antigenic material, e.g. terminal or side chain NH_2 or COOH groups, or -OH, -SH or other groups, with the functional group of the modified carrier, thus obtaining conjugates with peptide, ester, amine, disulfide, etc., covalent bonds.

The preferred carrier is silica which carries a side chain with a terminal aldehyde group, herein designated "aldehyde silica". Through reaction with a free

amino group of the toxin antigen, a Schiff base is formed ("silica-toxin imine") which is then reduced with sodium cyanoborohydride ($NaCNBH_3$) or sodium borohydride ($NaBH_4$) to give the silica-toxin conjugate of the invention, according to the following scheme :



The "aldehyde silica" is prepared as described by K. Ernst-Cabrera and M. Wilchek, 1986), according to the following scheme :



In an alternative procedure the starting material is a commercially available Diol silica (Lichrosorb® Diol).

Another alternative and preferred structure of the active substance of the invention, namely, the antigen or toxin protein or fragment thereof, is one in which the antigen, toxin protein or fragment thereof is covalently linked to a carrier protein such as bovine serum albumin (BSA) or thyroglobulin (TGB). One example is the

TGB-linked CTP3 peptide (TGB-CTP3) which is formed by first modifying the CTP3 by addition thereto of a p-aminophenylacetyl (papa) residue to form papa-CTP3 and then coupling the papa-CTP3 via an amide bond to TGB. The resulting TGB-CTP3 may then be coupled to any of the above noted inert carriers e.g., the aldehyde silica to form an Si-TGB-CTP3 conjugate to be used as a vaccine. Similarly, the other CTPs (CTP1, 2 and 4-6, and the CTB protein itself) may also be coupled to TGB and then conjugated to the inert carrier (eg. Si).

The vaccines comprising the conjugates of the invention in microparticulate form may be present in any form suitable for oral administration : suspension in an aqueous vehicle possible containing sodium bicarbonate, enteric capsules, tablets, coated tablets and the like. The capsules and tablets may be in sustained-release form, thus prolonging the period during which an effective concentration of the antigen is maintained in the intestines.

The inert carrier of the conjugates of the invention is essentially insoluble in the digestive tract and is mostly not absorbed, thus allowing presentation of the antigen in the intestines, where it will elicit antibodies against the target pathogen. Antibodies elicited in the intestine by such immunization will be mostly of the secretory IgA class (sIgA), and will appear in mucosal secretions of the body as well as in secretory fluids, such as human milk, saliva and tears. Thus, not only immunization against gastrointestinal and, more specifically, enteric infections, can be achieved with the vaccines of the invention, but also against pulmonary, respiratory, genitourinary and other infections.

Experiments have shown that a certain particle size range gives better results after oral administration. Particles in the range of up to about 10 microns give satisfactory results, but the preferred particle size range is from about 1 to about 25 microns, preferably 1-6 microns.

The dosage required for effective immunization will depend on the nature of the toxin and on the characteristics of the carrier. In some cases, an effective amount of 1 μg to 1000 μg per unit dosage form may be used. The vaccine may be

administered once or twice for effective immunization that will last several months, and then repeated as necessary.

One of the possibilities according to the invention is the production of multiple or mixed vaccines, for immunization against two or more infecting agents, wherein toxins, adhesins or fragments thereof derived from two or more different pathogens are covalently linked to identical or different inert carriers and the mixture is suspended in distilled water or other suitable liquid or made into tablets or capsules, and orally administered.

The invention will now be illustrated by the following non-limiting examples.

General Procedures and Materials

(a) **Chemicals.**

All organic chemicals, unless otherwise listed, were obtained from Sigma and inorganic chemicals from Aldrich. Organic solvents were of the highest grade available and were all obtained from Merck, Darmstadt, Germany, except for methanol, acetic acid, acetonitrile, acetone, ether, petroleum ether which were from Biolaboratories, Jerusalem, Israel. Purified cholera toxin B subunit and cholera toxin were kindly given by the Institute Merieux, France. Purity of all t-Boc-amino acids dissolved in dichloromethane were spotted on TLC aluminium silica sheets, chromatographed in methanol : chloroform (1:3 (v/v)), dried, exposed to trifluoroacetic acid (TFA) vapors for 5 min, sprayed with 1% (w/v) ninhydrin : acetone and then dried at 80°C for 5 min.

(b) **Solid phase peptide synthesis.**

The procedure of peptide synthesis was based on the standard Merrifield method (Merrifield, 1963). Two peptides, CTP3 and CTP1, from the cholera toxin B subunit, were first synthesized manually by the solid phase method, and later, these two peptides (CTP3 and CTP1) as well as peptides CTP2, CTP4, CTP5 and CTP6, also derived from the B subunit of the cholera toxin (see Jacob et a., 1983), were all synthesized on an automated peptide synthesizer. The resin support (Sigma, M-278) was mixed with the first N-protected t-butylcarbonyl amino acid at 1g resin per 1.5

mmol amino acid and then suspended with 5 ml absolute ethanol per 1g of resin and 0.14 ml anhydride triethylamine per mmol amino acid. The polymerization mixture was slowly stirred for three days in a reflux system at 80°C. The resin-amino acid conjugate was rinsed thrice with DMF, water and ethanol, dried and quantitated (10 mg) by the Moore-Stein analysis. The batch stepwise elongation of the peptide chain was DCC-mediated and HOBT-catalyzed with interspersing series of washings to remove excess reactants and by-products. Sometimes unreacted terminal amines were acetylated and binding completion was monitored with a ninhydrin assay by mixing the aminated resin with triketo-hydrindene and pyridine under a flame. The protecting groups were removed and the peptides cleaved from the resin by treatment with anhydrous HP containing 10% anisole and 1% 1,2-ethanedithiol as scavengers. They were then purified by gel-permeation chromatography in 5% acetic acid to remove contaminants and the purity of the crude product determined by analytical high pressure liquid chromatography and by automatic Stein-Moore analysis. Peptides were usually over 90% pure.

(c) **Coupling of peptides to protein carriers.**

For the coupling of peptides (e.g. the cholera toxin peptides CTP1-CTP6) to protein carriers, for example thyroglobulin (TGB) and bovine serum albumin (BSA), the peptides are first modified by standard methods to obtain p-aminophenylacetyl (papa)-bound peptides (see Spirer et al., 1977). The coupling of the modified peptides to the carrier proteins is by the following procedure, exemplified here for papa-CTP3 but also applicable for the other peptides : A 200 µl solution of 2N HCl containing 10 µmol papa-CTP3 was diazotized with 200 µl of 0.1N $NaNO_2$ for seven min at 4°C. The reaction mixture was checked on KI-starch paper, mixed with 2 µmol TGB or BSA dissolved in 2 ml 0.2N $NaHCO_3$ (4°C) and the pH adjusted to 9.0 with saturated K_2CO_3. The brown-red reaction mixture was dialyzed overnight at 4°C against 0.1N acetic acid, nanpoure water and lyophilized.

(d) **Maintenance of bacterial strains.**

V. cholerae 01 strain 395 was kindly provided by Myron M. Levine at the Center for Vaccine Development in Baltimore. Long-term storage was in glycerinated (15%) broth and, when in use, transferred about fortnightly onto fresh agar slants. Inocula were serially diluted in Luria-Bertani broth (1% (w/v) bacto-tryptone, 0.5% (w/v) yeast extract 1% (w/v) NaCl, pH 7.5) and 10 µl samples were spotted onto TCBS agar plates (Difco) and counted after overnight incubation at 37°C.

(e) **Protein estimation.**

Each protein sample was assayed according to the standard Lowry method (Lowry et al., 1951) and calibrated against a BSA standard curve.

(f) **Direct ELISA.**

The direct ELISA procedure was carried out in accordance with standard procedure (see Engvall and Perlmann, 1972). Briefly, flat-bottom 96-well polystyrene microtiter plates were coated with 100 µl/well antigen (37°C, 3 hrs), blocked with 200 µl of 3% BSA in PBS (1h, 37°C) and washed thrice with wash buffer (PBS/0.05% (v/v) Tween). Triplicate antibody samples (100 µl) with varying starting concentrations (serum 1:100, colostrum 1:50, intestinal secretions 1:50 and mouse lung lavage at 1:3) were reacted (37°C, 60 min) and washed thrice. Specific antibodies were screened (60 min, 37°C) with affinity purified goat peroxidase-labeled anti IgG or IgA antibody diluted in wash buffer. Unbound antibody was washed and fresh 2,2'-azino-bis(3-ethylbenz-thiazoline-6-sulfonic acid) (ABTS) substrate added to reveal the presence of the specific antibodies by a color reaction quantitated with an ELISA reader.

EXAMPLE 1. Preparation of the aldehyde silica.

Silica microparticles (Diol silica of microparticulate size 4.4-6.5 µm) containing vicinal diols (LiChrosorb® Diol, Merck, Darmstadt, Germany) were chemically modified into aldehydes by adding excess sodium metaperiodate into a stirred suspension of 1% (w/v) LiChrosorb® Diol in 10% acetic acid for two hours (e.g. 500 mg silica in 50 ml 10% acetic acid to which 500 mg sodium metaperiodate

was added while stirring). The presence of aldehydes was qualitatively ascertained before stopping the reaction or after completion of the reaction, by a hydrazono-de-oxobisubstitution assay. This assay consists of reacting the silica to a primary hydrazine derivative, 2,4-dinitrophenyl hydrazine, dissolved in ethanol or methanol. An instantaneous orange-colored precipitate appeared being an imine (Si-2,4-dinitrophenylhydrazone) which persists after several ethanol rinses. The aldehyde-silica was collected, washed (with double-deionized water) and dried by vacuum filtration in a standard filter holder unit having a filter of 0.45 μm porosity. The aldehyde-silica beads were resuspended in water, centrifuged and left to dry.

EXAMPLE 2. **Preparation of silica-cholera toxin B subunit conjugate.**

To 6 mg of cholera toxin B subunit (CTB) at 1 mg/ml in 0.1M phosphate buffered saline (PBS, pH 7.2), 100 mg aldehyde silica was added to form a suspension. The mixture was stirred for an hour. The aldehyde-silica underwent reductive amination, by the addition of the CTB, to form a simple imine or Schiff base. A CN-dihydro- addition reaction was performed to stabilize the bond between the aldehyde silica and the CTB, and this was done by adding an excess (60 mg) of sodium cyanoborohydrate portionwise. The mixture was stirred overnight. The reaction was stopped by washing the conjugate with deionized water until the optical density reached a plateau near zero. The silica-toxin conjugate was further washed with deionized water and 1M sodium chloride and dried under reduced pressure. By the Bradford protein assay, it was shown that the binding value was approximately 5 μg cholera toxin B subunit per 1 mg silica beads. By the more accurate Stein-Moore automated amino acid analysis (see Spackman et al., 1958) it was found that typical binding values were between 5 and 25.2 μg CTB per mg silica.

It should be noted that the stabilization of the conjugate with sodium cyanoborohydrate also serves to prevent oxo-de-alkylimino-bisubstitutions in the gastric acid environment, i.e. upon oral administration of the conjugates. Thus, the resultant insolubilized silica-protein conjugate contains a bond of high chemical and thermal stability.

Further, in theory, the average surface area available for binding is 500 m^2/g and the surface ligand coverage in LiChrosorb® Diol is 3,6 $\mu mole/m^2$ before any chemical modification. Thus, we have optimally an estimated 18.109 ligands per gram of silica. The conjugation chemistry provided high binding yields as estimated by amino acid analysis. There was batch to batch variability, but this was due to the different amounts of proteins or synthetic peptides used in separate experiments and not because of the actual binding which was usually quantitative.

With regards to the preparation of conjugates of the aldehyde-silica and the various peptides, a similar procedure to that described above was carried out. However, before conjugation to the aldehyde-silica, the peptides were either dissolved in 1:4:95 (v/v/v) triethylamine, acetic acid and water (pH approx. 6.0), PBS (pH 7.2) or 5% (w/v) sodium bicarbonate. The quantitative determination of silica conjugated to the peptides was also by the Stein-Moore amino acid analysis procedure. The binding values of the peptides to the silica were essentially as noted above for the CTB, although in some batches, e.g. that of a conjugate containing peptide CTP3 (50-64), the binding values were lower, namely, a batch of CTP3-containing conjugate was shown to have a binding value of 2.5 μg CTP3/mg silica. Other batches of CTP3 conjugate as well as the other peptide conjugates had values within the 5-25.2 μg per mg silica range.

EXAMPLE 3. **Immunization of rabbits.**

The products of Example 2 were evaluated as to their capability to elicit a secretory IgA (sIgA) response from the mucosal immunity by using the Thiry-Vella ileal loop model in the rabbit (Keren, et al., 1975). This model consists in surgically isolating an ileum segment (15-20 cm) with its vascular system intact and including a Peyer's patch. Ileal continuity is re-established by resection with an end-to-end anastomosis. To each end of the isolated ileal segment, a silastic tubing is attached and both tubings are brought through the midline abdominal incision and tunneled subcutaneously to the nape of the neck, where they are exteriorized and secured. The

peritoneum, muscles and skin are closed. Intestinal secretions can then be collected daily.

Rabbits were immunized intra-luminally with the silica-bound cholera toxin B subunit conjugate of Example 2. Antibody formation was detected by the 9th day as shown in Fig. 1 (Mean net optical density (O.D.) in two rabbits tested for IgA by ELISA in secretions (open circles) from the Thiry-Vella ileal loops and in serum (closed circles). Both rabbits were given a single dose of 0.3 mg cholera toxin B subunit bound to 250 mg Diol/silica.

In another experiment, female rabbits were immunized one day after mating with the conjugate silica-cholera toxin B subunit derived synthetic peptide CTP3 (5 mg of peptide bound to 250 mg silica particles) and then two more times at one week intervals during gestation. After parturition, colostrum was collected, cleared of fat and cells and tested for IgA by solid phase ELISA. The results show that the silica-peptide conjugate has triggered a mucosal response against the native cholera toxin.

These results as regards formation of secretory IgA antibodies in animals vaccinated with the conjugated antigens of the invention are indicative of the efficacy of such preparations.

EXAMPLE 4. **Immunization of rabbits with CTB and CTB-silica conjugates.**

In another set of immunization experiments, the capability of cholera toxin B subunit immobilized to silica particles to elicit a secretory response from the mucosal immunity system was also evaluated by using the Thiry-Vella ileal loop model in two groups of three rabbits. Each group was immunized with either CTB (0.5 mg) or CTB-silica conjugate (Si-CTB, 0.5 mg protein per conjugate) and ileal loop secretions were collected over a seventy day period. In these experiments the following procedures were performed :

(a) **Preparation of isolated Thiry-Vella ileal loops.**

'Pathogen-free' New Zealand albino rabbits (3 kg) were fasted for 24 hrs prior to surgery, with water allowed *ad libitum*. The rabbits were anesthetized intramuscularly (i.m.) with 0.8 ml/kg of 1:3:1 (v/v/v) 2% Rompun® (Bayer), 10%

Vetalar (Parke-Davis), 1% Combelen® (Bayer) and intravenously (i.v.) via infusion (Venothin® 25, Labomed) when needed. A wide spectrum antibiotic (100 mg/kg Keflin®) was injected i.v. to build up peak tissue levels and operative fluid therapy was given at 10 ml of 9% NaCl + 5% glucose per kg body weight per hr, while exposed organs in wait were kept moist with saline-soaked gauze.

The shaved, polydine-asceptised abdominal incision area of the unrestrained rabbit was dressed with sterile fenestrated, corner and 3M adhesive drapes to reduce contamination during the procedure. A 6-8 cm ventral midline was incised over the linea alba through the skin, abdominal musculature and peritoneum to reach the peritoneal cavity. The small intestine was located, the intestines externalized until locating a 20 cm long ileum segment enclosing a Peyer's patch (or two) about 30 cm proximal to the ileocecal valve. The communicating branches of the terminal vasa recta arterial arcadial vessels along the mesenteric borders, where the incisions will occur, were permanently ligated with 5/0 silk to prevent hemorrhage while the outside extremities of the isolated intestinal segment were clamped to prevent leakage of the ingesta onto the operative field. A transection was made along each of the serrefines distal from the future loop. The ends of the detached ileum segment with its vascular system intact, remained clamped shut and the mesentary was incised to allow its mobilization. The cut ends of intestine were placed opposing each other and ileal continuity was re-established as an end-to-end anastomosis. Mesenteric fat was trimmed back to the site of the ligated vasa recta as was the naturally everted mucosa. The cut edges of bowel were pulled together and silk 4/0 ligatures were tied snuggly with four throws at the mesenteric and anti-mesenteric extremities as stay sutures for traction. The mesenteric border was carefully freed of fat and the anchor threads gently pulled taut to stretch the two cut edges close together. The procedure was completed with 8-14 simple silk 5/0 interrupted sutures with external knots around the circumference of the intestine. A Lembert stitch was important at the mesenteric border since serosa was absent in this region. The lumen was checked for obstruction, leakage and bursting strength by injecting PBS into the proximal region of the

anastomose where there were few blood vessels and the fluid was then gently finger manipulated through the anastomose. The anchor threads were removed, the mesentery apposed using 3/0 interrupted silk sutures to prevent entanglement, avoiding the jejunal vessels, and the exteriorized organs returned into the peritoneal cavity.

A modified medical grade silastic tubing was tunneled through each end of the isolated ileal segments and fastened via purse-string sutures (previously, two thin layers of silastic® medical adhesive silicone type A were placed and allowed to cure around the circumference of the silastic tubing one cm apart). The loop was gently flushed with 70 ml saline, then air, to remove feacal and blood contents, and to test for leakage. The abdomen was closed after infiltrating (i.d.) the muscle layers with 1 ml Lidocain, passing the tubes through the muscles and finally the peritoneal and muscle margins were apposed with closely spaced continuous 2/0 silk sutures with both tubings anchored to the inner muscles. Benzylpenicillin G sodium (900,000 units) was provided i.p. and bamyxin ointment applied to the tube ends before exteriorization by passing subcutaneously to the nape of the neck through an incision. The skin was closed with a continuous 2/0 silk suture.

Post-surgical care consisted of giving 100 mg analgesic paracetamol and 0.5 ml (i.p. and i.m.) for at least 5 days of either Gentamicin (Keflin) or Cefazoin (Totacef). A collar was available if the rabbit tried to chew the tubing. Once the animal had defecated (24-48 hrs), a routine diet of pelleted food was resumed. Intestinal loop fluid secretions (2.4 ml) were collected daily by gently instilling air (20-35 ml), rinsing with 20 ml of sterile PBS and again air. Post-surgical progress was monitored carefully, with recorded complications of anorexia, peritonitis and possibly adynamic ileus. Rabbits that did not recover were put to sleep with an overdose of 0.5 ml/kg pentobarbitol sodium (Nembutal or Beutanasia).

(b) Histology. Free hand dissected ileum pieces 3 mm long were rinsed with a Krebs-Ringer solution, placed into Bouin for 48 hrs to preserve the sectioned tissues, dehydrated in 70% ethanol and stained with hematoxylin, eosin and light

green to reveal general cell structure. Histological sections were embedded by infiltration with paraffin.

(c) **Peroral immunization of rabbits, subsequent challenge of immunized rabbits with virulent *Vibrio cholerae* 01 and analysis by the ligated ileal loop assay.**

The peroral immunization and subsequent mating and milk collection and processing procedures were according to standard methods (see Yoshiyama and Brown, 1987). Briefly, control samples of serum and colostral fluids were taken before orogastric immunization, which was performed under anesthesia (1 ml of 3:2 (v/v) 10% Vetalar (Parke-Davis) and Acepromazine), several weeks before mating with a booster provided two weeks before parturition. The stomach gastric acid was neutralized with 150 mg cimetidine (i.m.) and 15 ml doses of 5% sodium bicarbonate, fed perorally thrice, at 15 min intervals, with the last containing the antigenic material, using either a nasogastric feeding tube or by intubation of the esophagus after placement of a plastic gag between the interdental space to keep the jaw open. Hypoperistalsis was obtained with a few drops of loperamide (Imodium®, Janssen). Peroral immunizations were carried out with an infant feeding tube. The primary immunizations were done twice at 10 day intervals in which 120 mg Si-CTB conjugate containing 0.3 mg CTB was administered. Control animals (2 groups) received either the silica (LiChrosorb® Diol) or the CTB alone. Subsequently the various groups of experimental animals were mated and the milk was collected and processed as follows : Matings were done simultaneously with deliveries of the litters occurring 30-32 days later. Colostrum and early milk were expressed daily, following parturition, from nursing, shaved rabbits by applying gentle pressure to the underlying tissue. The milk was diluted with an equal volume of chilled PBS with 0.1% sodium azide, centrifuged (15,000 g, 4°C, 4 hr) and the clear intermediate layer above the cell pellet and below the top lipid-oil layer was carefully withdrawn and stored at -20°C as aliquots. The colostrum was delipidized by adding 2% (w/v) aerosil 380 and stirred

for 4 hrs at 45°C. The polymer was then separated by centrifugation at 9,000 rpm and the supernatant filtered (0.8 μm, then 0.4 μm).

Challenge of the immunized and control animals by virulent *Vibrio cholerae* 01 and subsequent assay, by the standard ligated ileal loop assay, of the protection afforded by immunization was carried out as follows : Fifteen weeks after priming with either LiChrosorb® Diol, CTB or Si-CTB, anesthesized postweaned New Zealand White rabbits (4-8 months old, 1-2 kg.) were put on a liquid diet 48 hrs prior to the challenge assay. A 100 cm piece of small bowel beginning just above the appendix was exposed, kept moist while 6 cm experimental and 2 cm spacer loops were tied. Fully enteropathogenic *Vibrio cholerae* 01 strain 395 were slowly injected into the large loops in ten-fold serial dilutions in reverse order per pair with PBS as a control. A second tie was made to isolate the site of injection. 30 mls of 5% glucose was given i.p. to reduce postoperative dehydration, the abdomen closed and the skin clipped shut. Only water was supplied until the animals were sacrificed 18-20 hrs later. Pictures were taken, leakage between positive and negative loops was checked by injecting 0.1 ml of 2% Pontamine sky blue 6 BX and the fluid accumulated in the loops was measured by liberating the turbid contents from the tightly distended sacs. Animals having immunity to the pathogen should show very low levels of fluid accumulation in the loops.

The results of the above experiments are depicted in Figs. 2 and 3. In Fig. 2 there is shown graphically the results of the ELISA of sIgA (the dilution titer shown being 1:800) of ileal secretions from the two groups of rabbits which underwent Thiry-Vella surgical modification and which were primed with 0.5 mg CTB alone (open squares, control) or with 0.5 mg CTB linked to silica (Si-CTB, closed circles). For the purposes of accurately determining the amounts of sIgA in the collected ileal loop secretions and to reduce daily differences in the quantitation of sIgA obtained from immunized rabbit Thiry-Vella models, total sIgA was first approximated by sandwich ELISA and the ileal secretion concentrations equalized accordingly before determining the readings of the antigen specific sIgA titers (direct ELISA). As is

apparent from Fig. 2, over half the period of observation, there was a much greater amount of specific anti-CTB sIgA immunoglobulins in the ileal secretions of the CTB-immunized group, than in the Si-CTB-immunized group, even though they both were given approximately similar amounts of protein as determined by amino acid analysis. Secretory IgA antibody formation in the ileal secretions at a 1:800 dilution, was detected by the 9th day after priming, with a 0.1 mean absorbance above background taken at 600 nm in an ELISA reader. Thus, proteins bound to silica microparticles do retain their immunogenicity. Moreover, as demonstrated by the experiments on the persistence of silica in the intestinal tract (see Example 5 below), a greater amount of CTB-linked silica is ejected due to peristalsis. Thus, the greater titers observed must be due to the greater amount of CTB available at the onset. The temporal persistence of titers from the group of rabbits immunized with Si-CTB, may be explained by the greater temporal exposure of antigen to the mucosal immune system.

In Fig. 3 there is shown graphically the results of the challenge of rabbits immunized with Si-CTB (closed squares) and silica alone (closed circles, control) with the virulent strain 395 of *Vibrio cholerae* 01. The results are expressed as a dose-response curve, the experimental and control animals having been challenged with increasing amounts of the pathogen. As is apparent from Fig. 3, definite protection to the pathogen in the immunized animals was obtained for up to about 10^6 *Vibrio cholerae* 01 strain 395.

EXAMPLE 5. **Intestinal uptake and persistence of labeled silica particles.**

In order to determine the amount of silica absorbed in the intestines and the duration of the silica in the intestines a number of experiments were carried out using fluorescently and radiolabeled silica as well as radiolabeled Si-CTB conjugates. The procedures were as follows :

(a) **Preparation of fluorescently labeled silica particles.**

Fluorescently labeled silica microparticles were obtained by stirring 1.5 g aldehyde-silica into a 1M 1,2-diaminoethane solution at pH 9.0 for two hours,

washing with nanopure water and stirring into a solution containing 100 µg rhodamine B 200 sulfonyl chloride (derived from PC15 treated Lissamine-rhodamine B) for 5 hrs and again thoroughly washing.

(b) **Intestinal uptake of fluorescently-labeled silica.**

In three anesthesized male rabbits, 3-5 months old, about 100 mg silica-rhodamine suspended in PBS was injected into 3 cm long ileum segments, enclosing identifiable Peyer's patches, and left undisturbed 45, 60 and 90 min long, after which the rabbit was sacrificed. The Peyer's patches were cut out, rinsed, fixed in Bouin and cut by microtome. Some histological sections were stained with hematoxylin, eosin and green. The control consisted of histological sections from a non-injected rabbit.

(c) **Radioiodination of CTB.**

The iodination of CTB was done with the standard chloramine-T methods which consisted of dispensing an aliquot of 20 µCi $Na^{125}I$ into 1.2 ml of 1 µg/µl CTB in PBS at pH 7.5. Chloramine-T (80 µg in 40 µl) was then added and the mixture allowed to react (3 min), after which sodium metabisulphate (80 µ in 40 µl) was added (3 min) to convert iodine to iodide, thus preventing further iodination. The radioiodinated CTB was separated from the reactants by gel filtration using a 13 ml Sephadex G-25 gel bed equilibrated with BSA-PBS.

(d) **Intestinal persistence of CTB linked to silica.**

Two groups of three rabbits were gavaged with ^{125}I-CTB and ^{125}I-CTB-Si, respectively. The stomach's gastric acid was neutralized with two orogastric doses of 5% sodium bicarbonate given at 15 min intervals under anesthesia using a nasogastric feeding tube and injecting i.m. 150 mg cimetidine. The radiolabeled doses were then delivered i.g. and the rabbits allowed to feed and drink *ad libitum*. Twice a day, at exact intervals, faecal pellets were collected and counted in a gamma-counter. At the end of the experiment organ samples were counted. The background level was subtracted from an average value of individual faecal pellets collected before the start of the experiment.

The results of the above experiments are depicted in Figs. 4 and 5. In Fig. 4 there is shown a fluorescence micrograph of a Bouin-fixed, unstained intestinal section of the Peyer's patches showing characteristic embedded microparticle-like structures, i.e. numerous fluorescent dots. These data were observed in most of the twelve tissue sections obtained from different intestinal parts exposed at different times to the silica-rhodamine conjugates, and were either well separated or in aggregates. All fluorescent dots were of similar size and are believed to represent silica taken up by the intestinal tissue. The control was one rabbit sacrificed at the time of the experiment which did not show any characteristic bead-like fluorescence in either the Peyer's patches or the adjacent intestinal regions.

In Fig. 5 there is shown graphically the results of the persistence of CTB in the intestines. Animals given radiolabeled (^{125}I)-CTB are shown by way of hatched bars and those given radiolabeled ^{125}I-Si-CTB conjugates are shown by way of solid bars. From Fig. 5 it is apparent that both groups of animals shed the radioactivity quite differently.

Few counts were shed in faecal pellets of animals given the non-silica bound radiolabeled protein (^{125}I-CTB; approx. 10.15%). It is assumed that most of it was broken down and taken up by the nutritional system. Most counts were shed at day 3-5 post-gavage, possibly as metabolites. At day 9, shedding stopped (less than 0.1%).

In the animals given the silica-bound radiolabeled toxin (^{125}I-Si-CTB), a much larger amount of counts were shed (approx. 18.43%), mostly at days 2, 3 and 4 post-gavage. Persistence of shedding was observed even at day 7. After six days, the rabbits given ^{125}I-Si-CTB shed almost four times the amount of radioactivity than the control group. The initial burst of large and rapid shedding of counts is explained by the fact that most silica particles were removed by the organism by peristalsis. Also, intestinal tissue and especially Peyer's patches were shown to contain twice as many counts in the rabbit that was gavaged with the silica-bound radiolabeled protein. These results support our other data (not shown) about the long term persistence of silica bound proteins.

EXAMPLE 6. **X-Ray microanalysis of silica in Peyer's patch areas.**

The X-ray microanalysis procedure was carried out according to standard procedures (see Shuman et al., 1976). Briefly, three male rabbits were given i.n. 120 mg LiChrosorb® conjugated to a mixture of CTB peptides, i.e. CTP1-CTP6. Twenty hours later, the Peyer's patches were excised, rinsed with cold PBS, placed overnight at 4°C into freshly prepared 0.08M phosphate buffer including 2.4% glutaraldehyde and 0.8% paraformaldehyde, then dehydrated with alcoholic solutions of graded concentrations (25%, 50%, 75%, 95%, 100%) and purging with liquid carbon dioxide to the critical point (10 min). The dried Peyer's patches were trimmed by scratching off the villi until clearing of the lymphoid follicle bulges. The specimens were mounted with carbon paint and oriented with the follicle surface facing the edge of the carbon stub. Alternatively, thin slices of tissue were placed without staining on carbon stubs. Both were carbon coated using a Denton carbon evaporator before their analysis with a Phillips scanning electron microscope. The specimens were analysed using energy dispersive X-ray analytical equipment and the data collected and processed by a standardless semiquantitative (SSQ) analysis system. Morphology of the specimens was recorded by photographing the secondary image from the recording cathode ray tube. Micrographs were taken at 2000 scan lines per frame with an exposure time of 32 seconds.

The results of the X-ray microanalysis are shown in Figs. 6 and 7. In Fig. 6 there is shown a scanning transmission image of a fixed, unstained section of an ileal section showing characteristic embedded microparticle structures. The bisecting lines point to the area of electron probe X-ray microanalysis. It should be noted that the analysis of the surface areas of the lymphoid follicle bulges of the Peyer's patches by electron probe X-ray microanalysis did not allow any detection of silica within the tissue. This is easily explained by the fact that the instrument cannot penetrate further than 10 μm. Some microparticles which were not cleared by the intestinal peristalsis and somehow fixed to the tissue were identified by the electron beam as silica. Thus,

thin slices of similar tissue were used with a preparation similar to that of the fluorescent silica uptake experiment, these being the ones depicted in Fig. 6.

In Fig. 7 there is shown the energy spectrum from a single embedded microparticle-like structure showing only one elemental constituent, namely, silica (left panel); and from another site (right panel) taken from the surrounding organic matrix of the thin slice material. It should be noted that the computer analysis of the X-ray spectra allowed elemental microanalysis of visible embedded microparticles. These particles were difficult to locate within the tissue. When the beam was focused on a bead-like structure (bisecting lines in Fig. 6), silica was the exclusive atom detected (left panel, Fig. 7) and when the beam was directed to the surrounding tissue, no silica was detected (right panel, Fig. 7). Actually, no other metals were detected in even very low quantities which is unlike what is known in physiological tissues. This is explained by the rough fixation procedure and by the fact that ultrathin secretions using cryo conditions would then be required.

EXAMPLE 7. **Immunization of mice with silica-bound TGB-CTP3.**

The mice were immunized and assayed according to the standard mouse lung lavage procedure. Briefly, *M. musculus* with different H-2 backgrounds (Balb.K, B10.S, B10.BR, C57BL/10J, B10A, SJL/J, Balb.B, Balb/C) were immunized i.g. with TGB-linked Si-CTP3 conjugates or with TGB-linked CTP3 (controls), at about 15 weeks of age. Immunizations were with an equivalent of 50 µg CTP3 per mouse. Both primary and booster immunizations, at fifteen day intervals, were performed. They were asphyxiated by CO_2 suffocation (dry ice), placed on their back and swabbed with 70% ethanol. A midline ventral incision was made and the fur stretched away from the abdomen to the mandible, uncovering the muscle layer. The sternohyoid and throat muscles were teased apart, the trachae exposed and left *in situ*. The thoracic cage was partially removed to uncover the intact lungs and a thread placed just under the trachae to slightly pull it up. A Venflon® I.V. cannula (Vigo, Sweden) was inserted into the trachae towards the lungs 5 mm from the larynx and the injection valve (Ø18G/1.2mm O.D. 45mm) slowly pulled out, leaving the Teflon

catheter inserted. The cannula was secured to the trachae with a single silk knot and connected to a three-way stopcock, previously attached to two 5 ml syringes, one empty and the other containing 0.1% BSA in PBS (pH 7.2). Three 700-800 µl lung lavages drew bronchoalveolar secretion aspirate, which were collected pooled, aliquoted and stored at -20°C. Serum was obtained by cutting a nearby artery and the heart. Just before use, the washings were subjected to centrifugation to remove cellular debris and pooled samples containing erythrocytes were discarded. The lung lavage secretions and the serum were collected a week after the booster immunization.

The results of the above immunizations are depicted in Figs. 8 and 9. Fig. 8 shows the results of the ELISA (using BSA-bound CTP3 to eliminate cross-reaction with TGB) of sIgA from lung lavage secretions obtained from three C57BL/10J mice immunized with Si-TGB-CTP3 (open squares) or with TGB-CTPP3 (closed circles). Fig. 9 shows the results of the ELISA (using BSA-bound CTP3) of IgG from serum obtained from five SJL/J mice immunized with Si-TGB-CTP3 (open squares) or with TGB-CTP3 (closed circles). Thus, from Figs. 8 and 9 it is apparent that a high CTB responder mouse (SJL/J), when primed and boosted once each with thyroglobulin-bound CTP3 linked to silica (Si-TGB-CTP3), gave better anti-CTB titers in the serum than when the antigen complex was used unlinked to silica (Fig. 9). Other strains of mice were used (Balb.B, B10A, Balb.K, B10.BR, B10.S and Balb/C), but these did not give any anti-CTB titers either in the serum or lavage secretions. Greater anti-CTP3 titers from Si-TGB-CTP3 immunized mice were observed from the lavage secretions (sIgA) in only one strain of mouse, C57BL/10J (Fig. 8).

REFERENCES

1. Black R. et al., International Symposium on Enteric Infections in Man and Animals : Standardization of Immunological Procedures, Dublin, Ireland, 1982.
2. Black, R. et al., Develop. Biol. Standard Vol. 53, pp. 9-14 (S. Karger, Basel) 1983.
3. Edelman, R., Vaccine, Vol. 7, pp. 166-167, 1989.
4. European Patent Application EP 305968 (March 8, 1989).
5. Engvall, E. and P. Perlmann, Enzyme-linked immunoabsorbent assay, ELISA. III. Quantitation of specific antibodies by enzyme-labeled anti-immunoglobulin in antigen-coated tibes. J. Immunology, 109:129-135, 1972.
6. Ernst-Cabrera, K. and M., Wilchek, Analytical Biochemistry, 159 : 267-272, 1986.
7. Ernst-Cabrera, K. and M. Wilchek. Trends in Anal. Chem. Vol. 7, pp. 58-63, 1988.
8. Jacob, C.O., M. Sela and R. Arnon. Antibodies against synthetic peptides of the B subunit of cholera toxin : cross-reaction and neutralization of the toxin. Proc. Natl. Acad. Sci. USA 80 : 7611-7615, 1983.
9. Keren, D.F., H.L. Elliott, G.D. Brown and J.H. Yardley. Atrophy of villi with hypertrophy and hyperplasia of paneth cells in isolated (Thiry-Vella) ileal loops in rabbits. Gastroent. 68 : 83-93, 1975.
10. Levine, M.M. et al., Infection and Immunity, Vol. 21, No. 1, ppp. 158-162, 1978.
11. Levine, M.M., J.B. Kaper, D. Herrington, G. Losonsky, J.G. Morris, M.L. Clements, R.E. Black, B. Tall and R. Hall. Volunteer studies of deletion mutants of *Vibrio cholerae* 01 prepared by recombinant techniques. Infect. Immun. 56 : 161-167, 1988.
12. Levine, M.M. et al., The Lancet, August 27, pp. 467-476, 1988.
13. Lowry, O.H., N.J. Rosenbrough, A.L. Farr and R.J. Randall. Protein measurement with the Folin phenol reagent. J. Biol. Chem. 193 : 265-275, 1951.

14. Mascart-Lemone, F. et al., Scand. J. Immunol., Vol. 28, pp. 443-448, 1988.

15. Merrifield R.B. Solid phase peptide synthesis. I. The synthesis of a tetrapeptide. J. Amer. Chem. Soc. 85 : 2149-2154, 1963.

16. Shuman, H., A.V. Somiyo and A.P. Somiyo. Quantitative electron probe microanalysis of biological thin secretions : methods and validity. Ultramicroscopy, 1 : 317-339, 1976.

17. Spackman, D.H., W.H. Stein and S. Moore. Automatic recording apparatus for use in the chromatography of amino acids. Anal. Chem. 30 : 1190-1206, 1958.

18. Spirer, Z., V. Zakuth, N. Bogair and M. Fridkin. Radioimmunoassay of the phagocytosis-stimulating peptide tuftsin in normal and splenectomized subjects. Eur. J. Immun. 7 : 69-74, 1977.

19. Yoshiyama, Y. and W.R. Brown. Specific antibodies to cholera toxin in rabbit milk are protective against *Vibrio cholerae*-induced intestinal secretion. Immunology 61 : 543-547, 1987.

CLAIMS

1. A conjugate of an antigenic material selected from the group of a toxin, or fragment thereof, a toxoid and/or an adherence antigen derived from an infecting agent, which is covalently bound to a physiologically acceptable inert carrier in microparticulate form.

2. A conjugate according to claim 1 wherein the carrier is silica, aluminum silicate, kaolin or latex.

3. A conjugate according to claim 1 or 2 wherein the inert carrier is chemically modified silica carrying functional groups such as OH, COOH, CHO, NH_2, SH and the like.

4. A conjugate according to any of claims 1 to 3 wherein the antigenic material is a bacterial toxin.

5. A conjugate according to claim 4 wherein the antigenic material is cholera toxin B subunit which is covalently bound to modified silica through an amino group.

6. A conjugate according to any one of claims 1-3, wherein the antigenic material is selected from any one of the cholera toxin B fragments of the group consisting of peptides CTP1, CTP2, CTP3, CTP4, CTP5 and CTP6.

7. A conjugate according to claim 6 wherein the antigenic material is the cholera toxin B fragment CTP3 of the formula :

Val-Glu-Val-Pro-Gly-Ser-Gln-His-Ile-Asp-Ser-Gln-Lys-Lys-Ala
50 60

8. A conjugate according to any one of claims 1-7, wherein said antigenic material is further characterized by being covalently linked to a carrier protein.

9. A conjugate according to claim 8 wherein said carrier protein is bovine serum albumin (BSA) or thyroglobulin (TGB).

10. A conjugate according the claim 9 wherein said antigenic material is the CTB peptide CTP3 which is linked to TGB.

11. A method for the production of a conjugate according to any of the preceding claims which comprises reacting the antigenic material with a physiologically acceptable inert carrier having suitable functional groups in microparticulate form and, if necessary, further reacting the obtained product in order to obtain said conjugate.

12. A method according to claim 11 for the production of a conjugate as claimed in claim 5 which comprises reacting cholera toxin B subunit with aldehyde silica of the formula :

/ -O-Si-$(CH_2)_3$-O-CH_2-CHO

and the resulting silica-toxin imine of the formula :

/-O-Si-$(CH_2)_3$-O-CH_2-CH=N-toxin

is reduced in the presence of $NaBH_4$ or $NaBH_3CN$ thus producing the silica-toxin conjugate of the formula :

/-O-Si-$(CH_2)_3$-O-CH_2-CH_2-NH-toxin.

13. A vaccine for oral immunization against an infecting agent comprising an effective amount of a conjugate according to claim 1.

14. A vaccine according to claim 13 for oral immunization against gastrointestinal microbial infections.

15. A vaccine according to claim 13 or 14 for oral immunization against cholera comprising the conjugate according to claim 5 or 6.

16. An oral mixed vaccine for simultaneous immunization against several infecting agents comprising two or more suitable conjugates according to claim 1.

17. A method for oral immunization against infecting agents which comprises the administration of an effective amount of a suitable vaccine as claimed in any of claims 13 to 16.

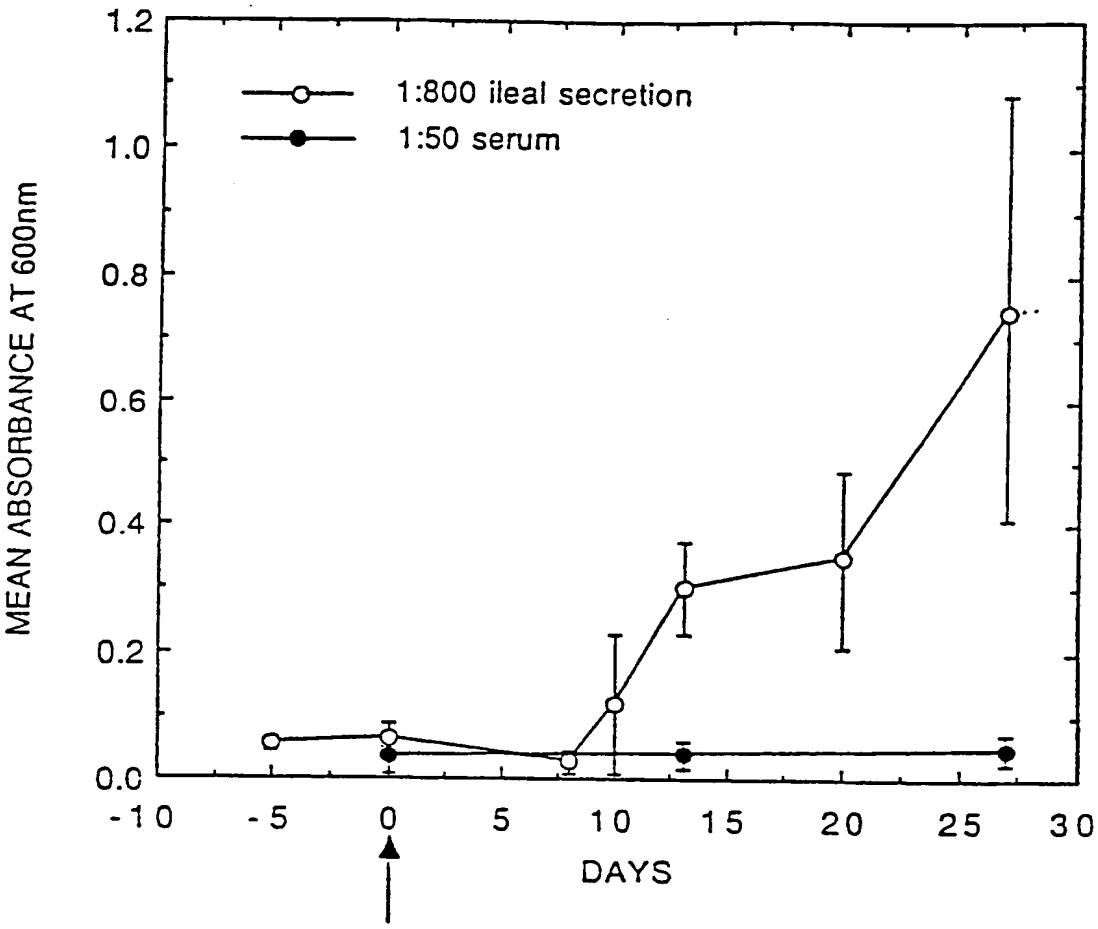
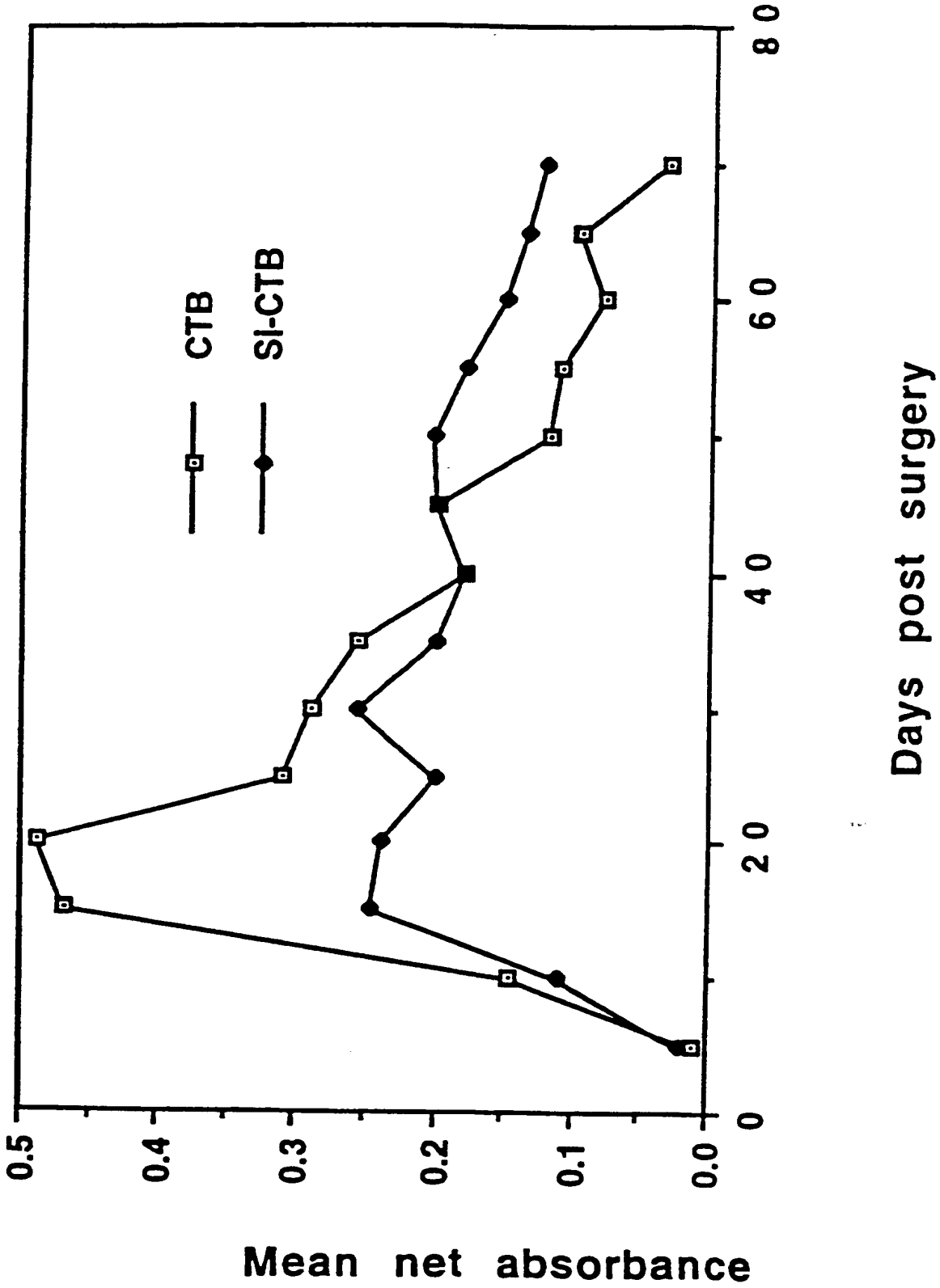

1.2
1.0
0.8
0.6
0.4
0.2
0.0
MEAN ABSORBANCE AT 600nm
1:800 ileal secretion
1:50 serum
-10
-5
0
5
10
15
20
25
30
DAYS

Figure 1


CTB
SI-CTB
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0.3
0.2
0.1
0.0
0
20
40
60
80
Mean net absorbance
Days post surgery

Figure 2

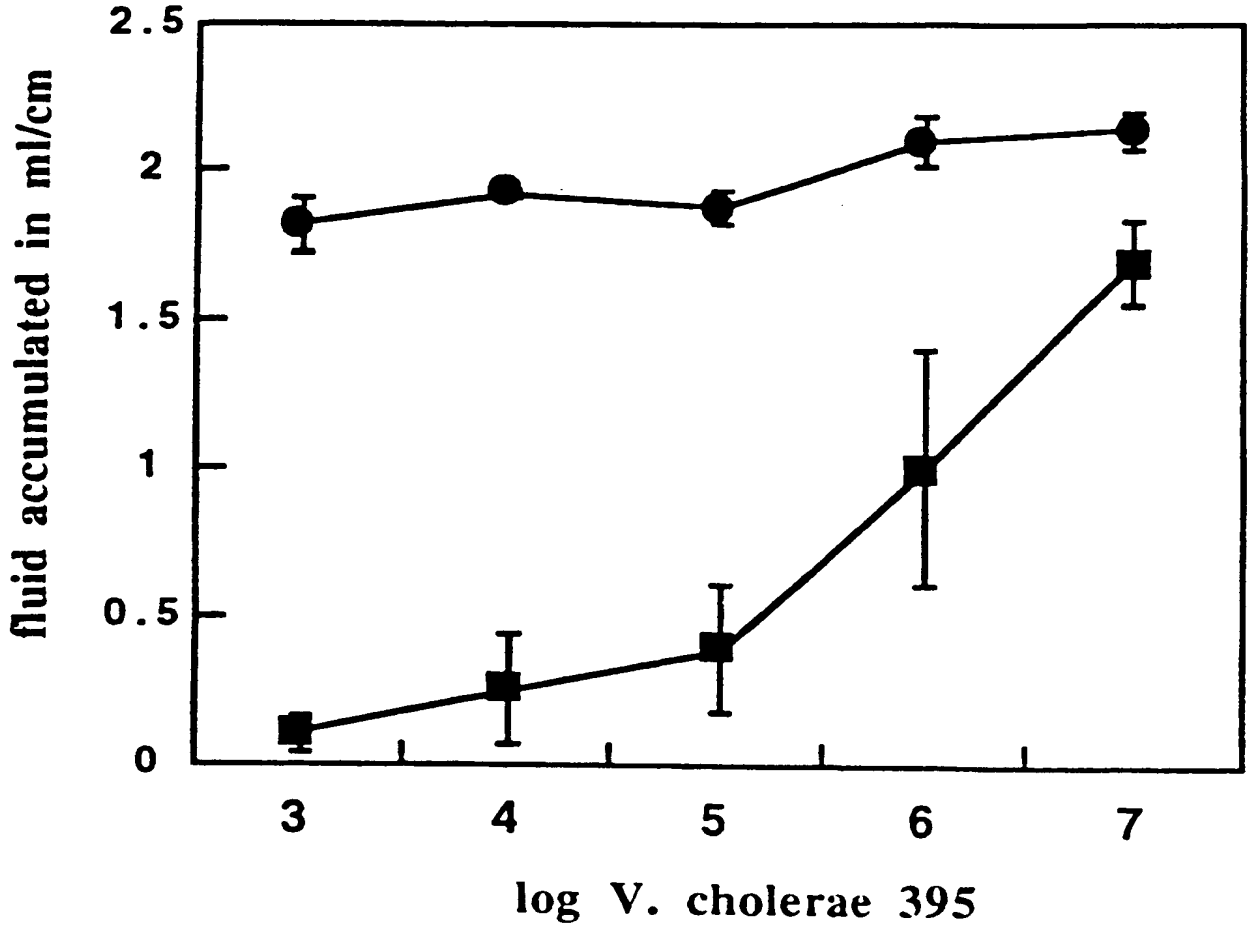
2.5
2
1.5
1
0.5
0
fluid accumulated in ml/cm
3
4
5
6
7
log V. cholerae 395

Figure 3

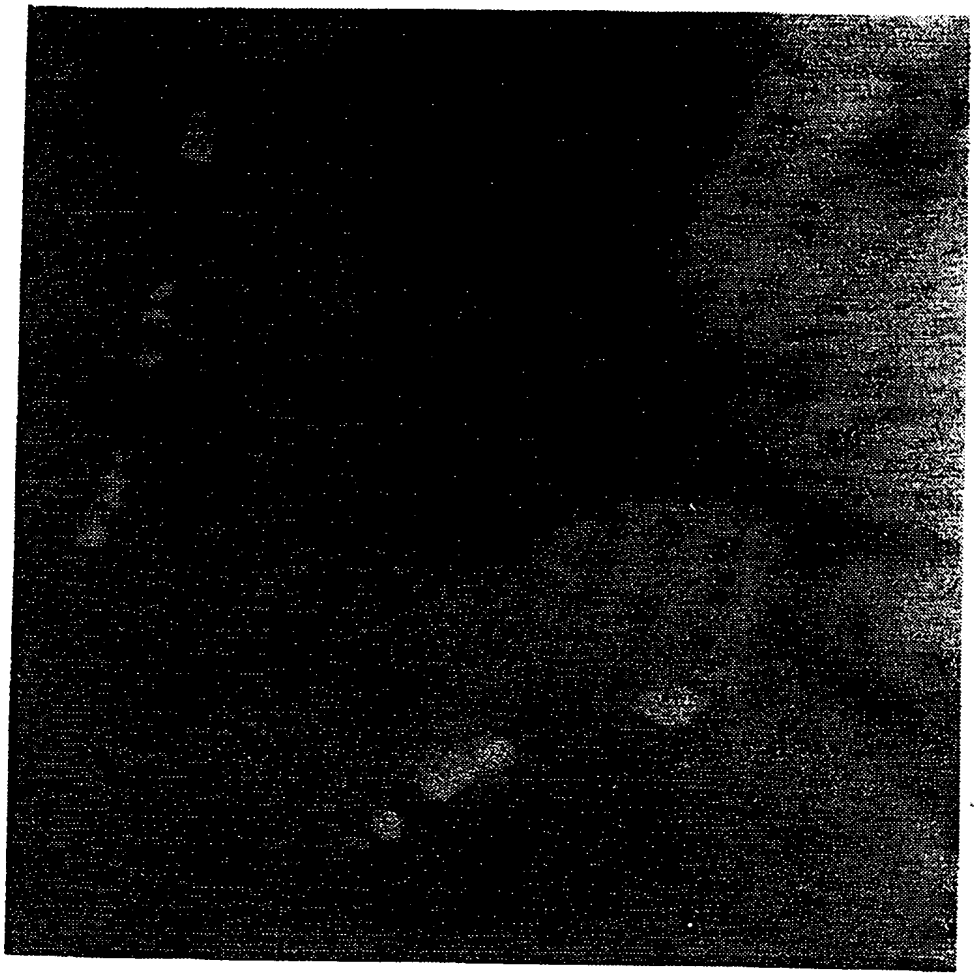

Figure 4

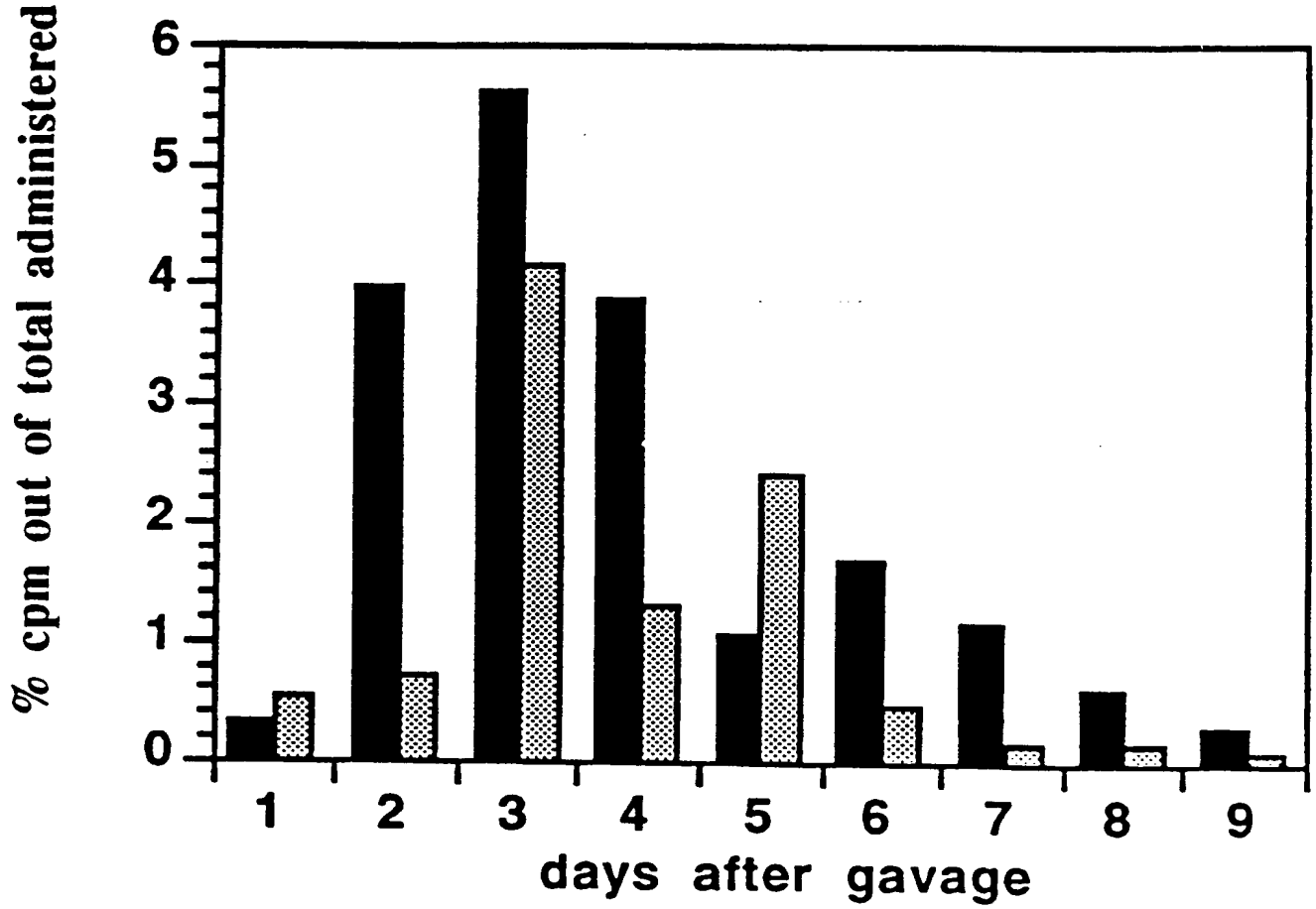
% cpm out of total administered
0
1
2
3
4
5
6
1
2
3
4
5
6
7
8
9
days after gavage

Figure 5

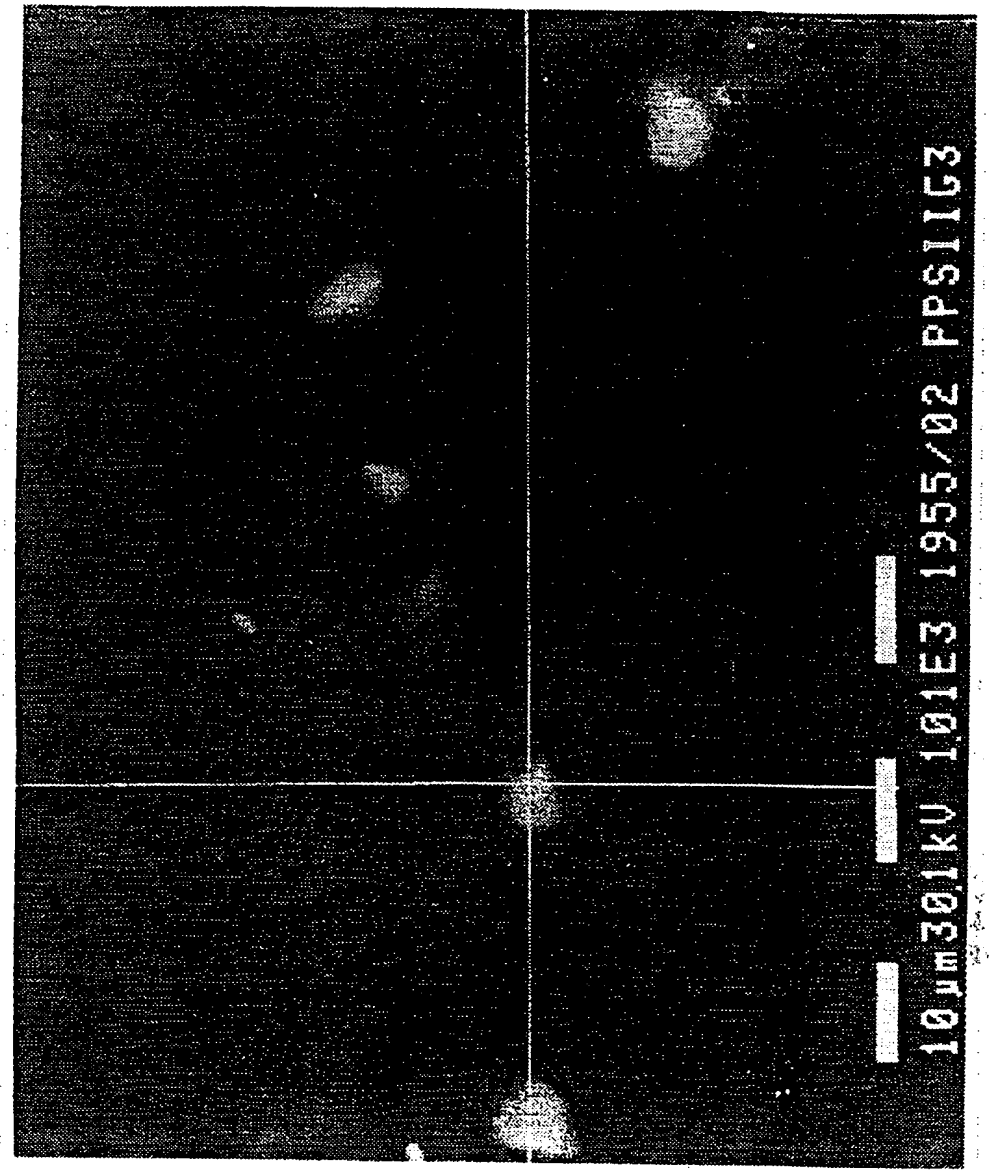

Figure 6

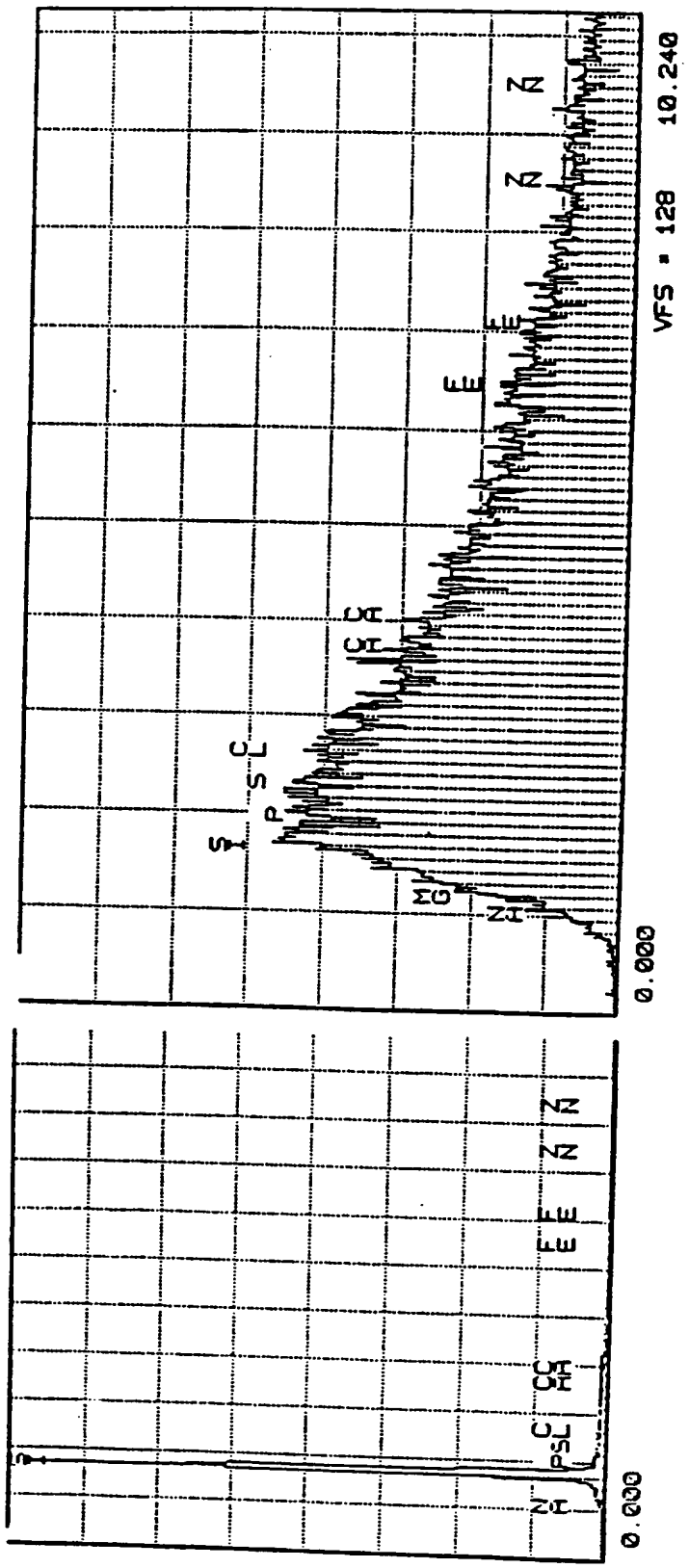

Figure 7

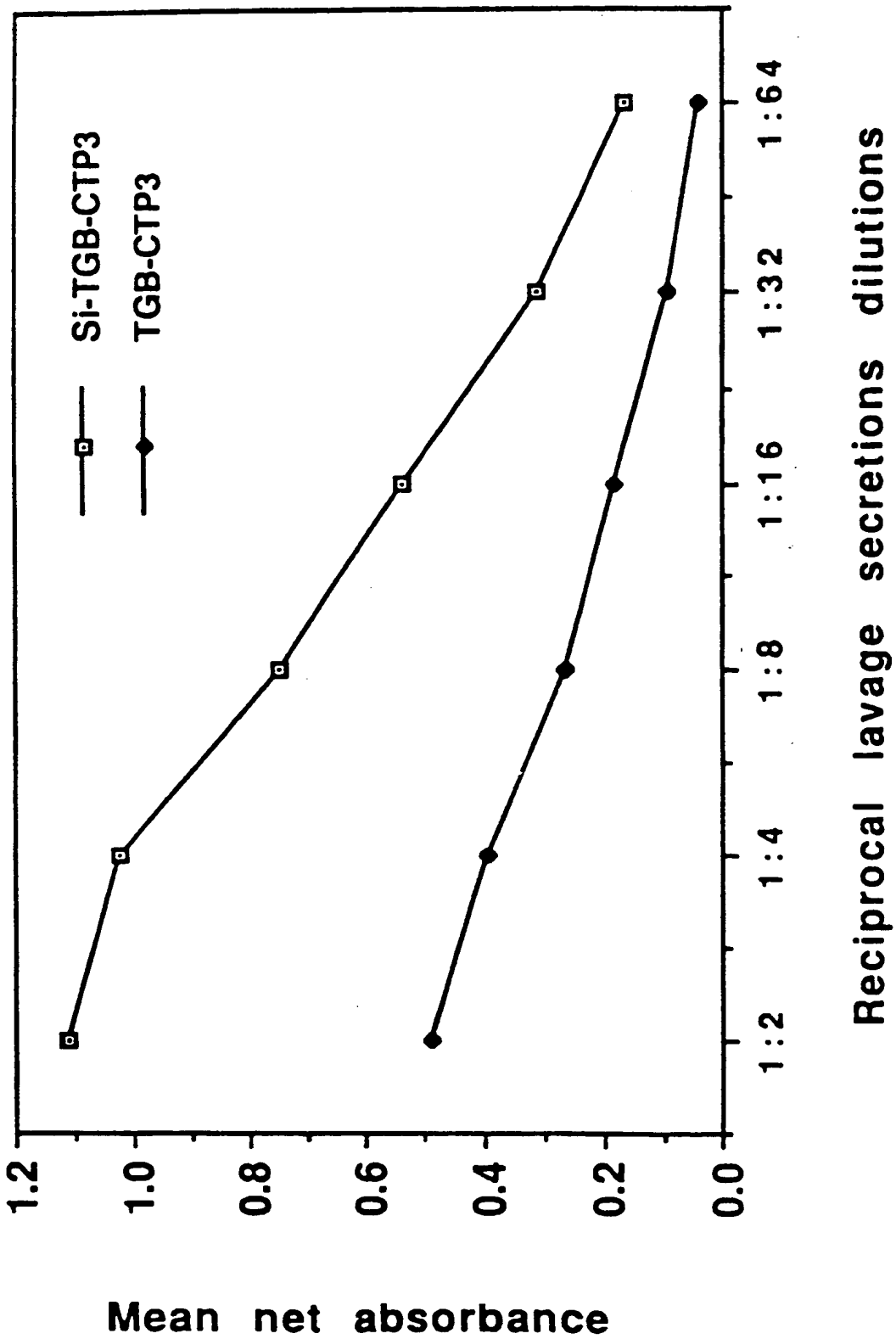
Si-TGB-CTP3
TGB-CTP3
1.2
1.0
0.8
0.6
0.4
0.2
0.0
1:2
1:4
1:8
1:16
1:32
1:64
Reciprocal lavage secretions dilutions
Mean net absorbance

Figure 8

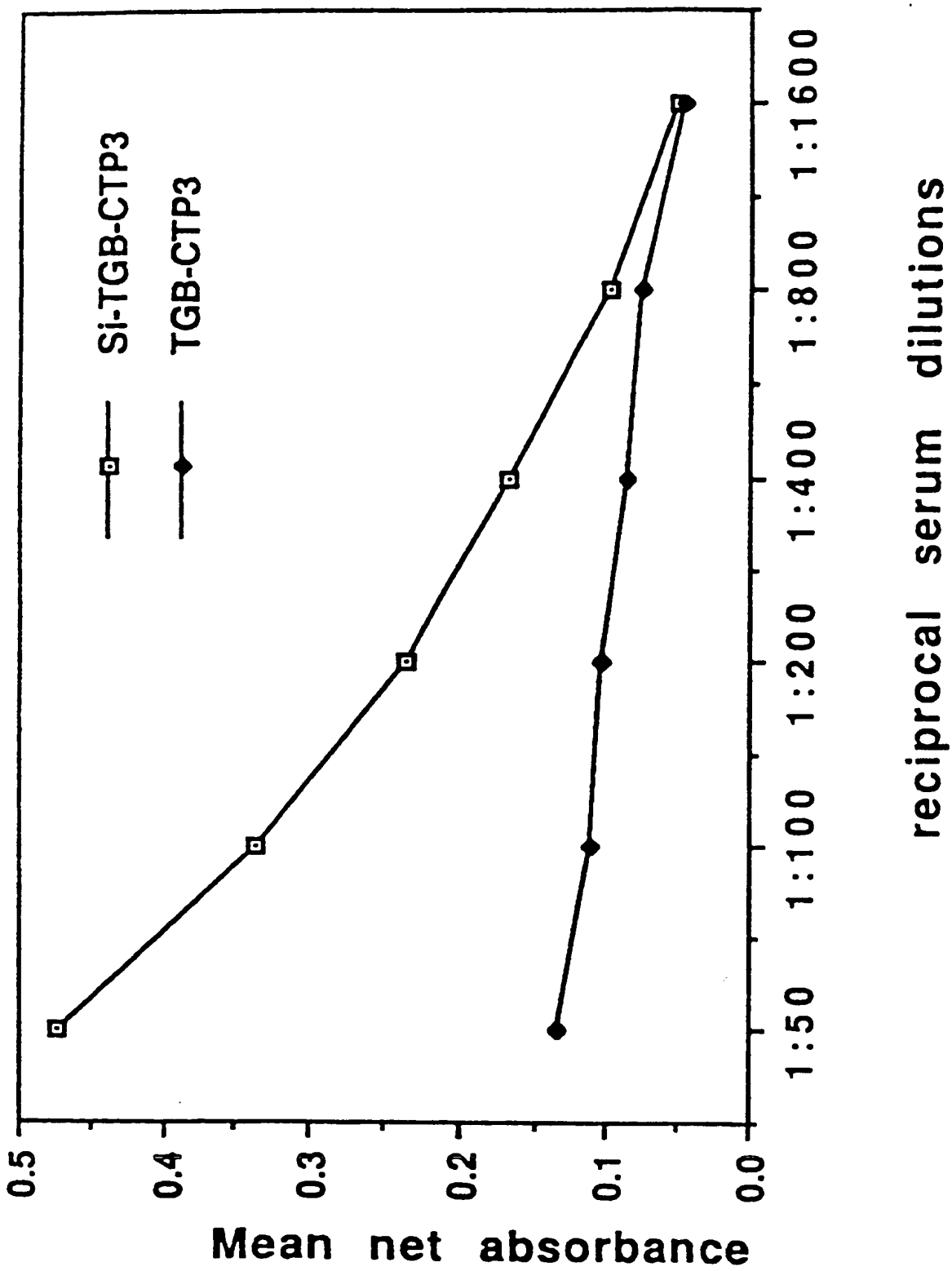
Si-TGB-CTP3
TGB-CTP3
0.5
0.4
0.3
0.2
0.1
0.0
Mean net absorbance
1:50
1:100
1:200
1:400
1:800
1:1600
reciprocal serum dilutions

Figure 9

INTERNATIONAL SEARCH REPORT

International Application No
PCT/EP 95/01661

A. CLASSIFICATION OF SUBJECT MATTER
IPC 6 A61K39/385 A61K47/48

According to International Patent Classification (IPC) or to both national classification and IPC

B. FIELDS SEARCHED

Minimum documentation searched (classification system followed by classification symbols)
IPC 6 A61K C07K

Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched

Electronic data base consulted during the international search (name of data base and, where practical, search terms used)

C. DOCUMENTS CONSIDERED TO BE RELEVANT

Category *	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
Y	FR,A,2 550 943 (YEDA RESEARCH AND DEVELOPMENT) 1 March 1985 see the whole document	1-17
Y	EP,A,0 305 968 (YEDA RESEARCH AND DEVELOPMENT) 8 March 1989 cited in the application see the whole document	1-17

☐ Further documents are listed in the continuation of box C.

☒ Patent family members are listed in annex.

* Special categories of cited documents :

"A" document defining the general state of the art which is not considered to be of particular relevance

"E" earlier document but published on or after the international filing date

"L" document which may throw doubts on priority claim(s) or which is cited to establish the publication date of another citation or other special reason (as specified)

"O" document referring to an oral disclosure, use, exhibition or other means

"P" document published prior to the international filing date but later than the priority date claimed

"T" later document published after the international filing date or priority date and not in conflict with the application but cited to understand the principle or theory underlying the invention

"X" document of particular relevance; the claimed invention cannot be considered novel or cannot be considered to involve an inventive step when the document is taken alone

"Y" document of particular relevance; the claimed invention cannot be considered to involve an inventive step when the document is combined with one or more other such documents, such combination being obvious to a person skilled in the art.

"&" document member of the same patent family

Date of the actual completion of the international search	Date of mailing of the international search report
6 September 1995	25. 09. 95

Name and mailing address of the ISA	Authorized officer
European Patent Office, P.B. 5818 Patentlaan 2 NL - 2280 HV Rijswijk Tel. (+31-70) 340-2040, Tx. 31 651 epo nl, Fax (+31-70) 340-3016	Moreau, J

Form PCT/ISA/210 (second sheet) (July 1992)

INTERNATIONAL SEARCH REPORT

:rnational application No.
PCT/EP 95/01661

Box I Observations where certain claims were found unsearchable (Continuation of item 1 of first sheet)

This international search report has not been established in respect of certain claims under Article 17(2)(a) for the following reasons:

1. [X] Claims Nos.: 17
because they relate to subject matter not required to be searched by this Authority, namely:
Remark: Although claim 17 is directed to a method of treatment of the human/animal body, the search has been carried out and based on the alleged effects of the compound/composition.

2. [ ] Claims Nos.:
because they relate to parts of the international application that do not comply with the prescribed requirements to such an extent that no meaningful international search can be carried out, specifically:

3. [--] Claims Nos.:
because they are dependent claims and are not drafted in accordance with the second and third sentences of Rule 6.4(a).

Box II Observations where unity of invention is lacking (Continuation of item 2 of first sheet)

This International Searching Authority found multiple inventions in this international application, as follows:

1. [ ] As all required additional search fees were timely paid by the applicant, this international search report covers all searchable claims.

2. [ ] As all searchable claims could be searches without effort justifying an additional fee, this Authority did not invite payment of any additional fee.

3. [ ] As only some of the required additional search fees were timely paid by the applicant, this international search report covers only those claims for which fees were paid, specifically claims Nos.:

4. [ ] No required additional search fees were timely paid by the applicant. Consequently, this international search report is restricted to the invention first mentioned in the claims; it is covered by claims Nos.:

Remark on Protest

[ ] The additional search fees were accompanied by the applicant's protest.

[ ] No protest accompanied the payment of additional search fees.

INTERNATIONAL SEARCH REPORT

Interna[illegible]pplication No
PCT/EP 95/01661

Patent document cited in search report	Publication date	Patent family member(s)		Publication date
FR-A-2550943	01-03-85	DE-A,C	3430894	14-03-85
		GB-A,B	2145419	27-03-85
		US-A-	4751064	14-06-88
EP-A-305968	08-03-89	NONE		